A Comparative Study of Fibrous Dysplasia and Osteofibrous Dysplasia with Regard to $G_S\alpha$ Mutation at the Arg[201] Codon

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis of Paraffin-Embedded Tissues

Akio Sakamoto,* Yoshinao Oda,*
Yukihide Iwamoto,[†] and Masazumi Tsuneyoshi*

From the Department of Anatomic Pathology, Pathological Sciences, and the Department of Orthopaedic Surgery,[†] Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

Fibrous dysplasia and osteofibrous dysplasia are both benign fibro-osseous lesions of the bone and are generally seen during childhood or adolescence. Histologically, the features of these bone lesions sometimes look quite similar, but their precise nature remains controversial. Mutation of the α subunit of signal-transducing G proteins ($G_S\alpha$), with an increase in cyclic adenosine monophosphate (cAMP) formation, has been implicated in the development of multiple endocrinopathies of the Albright-McCune syndrome and in the development of fibrous dysplasia. We studied $G_S\alpha$ mutation at the Arg[201] codon in seven cases of fibrous dysplasia (six monostotic lesions and one polyostotic lesion) and seven cases of osteofibrous dysplasia using formalin-fixed, paraffin-embedded tissue, by means of polymerase chain reaction-restriction fragment length polymorphism and direct sequencing analysis. All of the seven cases of fibrous dysplasia showed missense point mutations in $G_S\alpha$ at the Arg[201] codon that resulted in Arg-to-His substitution in three cases and Arg-to-Cys substitution in four cases. On the other hand, the seven cases of osteofibrous dysplasia and the normal bone used as a control showed no such mutation. These data suggest that fibrous dysplasia and osteofibrous dysplasia have different pathogeneses and that the detection of $G_S\alpha$ mutation at the Arg[201] codon is quite useful for distinguishing between these lesions.   (*J Mol Diag 2000, 2:67–72*)

Fibrous dysplasia is a benign intramedullary fibro-osseous lesion that can occur in any bone. Lichtenstein and Jaffe gave it the name "fibrous dysplasia" in two classic publications in 1938 and 1942.[1,2] Three forms of fibrous dysplasia are distinguishable: a monostotic form, a polyostotic form, and a polyostotic form associated with endocrinopathies and skin pigmentation, also known as the Albright-McCune syndrome.[3,4] Histologically, fibrous dysplasia is composed of slender and curved trabeculae of bone and a cellular proliferation of fibroblast-like cells, which are characteristically associated with long bones. Moreover, some cases of fibrous dysplasia also show sclerotic patterns, particularly in craniofacial bones.[5]

Mutation of the α subunit of signal-transducing G proteins ($G_S\alpha$) at the Arg[201] codon stimulating cyclic adenosine monophosphate (cAMP) formation has been identified in various tissues in the Albright-McCune syndrome[6,7] and is also thought to underlie the development of fibrous dysplasia associated with a cellular retraction and deposition of abnormal bone matrix led by increased cAMP formation.[5,8–11]

Osteofibrous dysplasia of long bone is a rare fibro-osseous lesion of unknown pathogenesis described as a distinct entity by Campanacci in 1976.[12] Osteofibrous dysplasia is an intracortical lesion which occurs almost exclusively in the tibia or fibula of children younger than 10 years of age and often presents as a painless enlargement of the tibia with anterior or anterolateral bowing. Histologically, osteofibrous dysplasia is characterized by woven bone trabeculae with a rimming of osteoblasts and a cellular proliferation of fibroblast-like cells, and has long been thought to be related to adamantinoma of long bones.[13–15]

Fibrous dysplasia sometimes resembles osteofibrous dysplasia histologically. Osteofibrous dysplasia has been

Table 1. Oligonucleotide Primers Used for PCR Amplification of $G_s\alpha$ Gene

Primer	Strand	Oligonucleotide sequence
$G_s\alpha$		
P1	Sense	5′-CCATTGACCTCAATTTTGTTTCAG-3′
P2	Antisense	5′-GGTAACAGTTGGCTTACTGGAAGTTG-3′
P3*	Sense	5′-TTTTGTTTCAGGACCTGCTTCGCGGC-3′
P4	Antisense	5′-ACTTTGTCCACCTGGAACTTGGTCTC-3′
p53 Exon 8	Sense	5′-TGGTAATCTACTGGGACGGA-3′
	Antisense	5′-GCTTAGTGCTCCCTGGGGGC-3′

*Underlining indicates a new restriction site in the normal allele for *Eag*I.

considered to be a congenital lesion or a variant of fibrous dysplasia.[16] However, the precise nature of fibrous dysplasia and osteofibrous dysplasia remains controversial. Though $G_s\alpha$ mutation in cases of fibrous dysplasia has been extensively studied, such mutation in osteofibrous dysplasia has not been fully evaluated.

In this study, we investigated the occurrence of $G_s\alpha$ mutation at the Arg[201] codon in both fibrous and osteofibrous dysplasia with a view to verifying whether the presence or absence of this mutation can help to distinguish between these two lesions.

Materials and Methods

Specimens

From the histopathology files at our institute, formalin-fixed, paraffin-embedded tissue blocks of seven cases of fibrous dysplasia and seven cases of osteofibrous dysplasia, which had been decalcified in hydrochloric acid for a maximum of 3 days, were used for this study. The seven cases of fibrous dysplasia comprised six monostotic lesions and one polyostotic lesion. None of the patients with fibrous dysplasia exhibited Albright-McCune syndrome. One mature bone specimen obtained from a donor with neither fibrous dysplasia nor osteofibrous dysplasia was used for a comparative control.

Formalin-Fixed, Paraffin-Embedded Tissue DNA Extraction

DNA was extracted from a 30-μm paraffin-embedded tissue section as follows. Paraffin was removed with xylene, and then the sample was washed twice with 100% ethanol and subsequently dried. The tissue was suspended in digestion buffer (100 mmol/L sodium chloride, 10 mmol/L Tris-hydrochloric acid, 25 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate) containing 10 μg proteinase K and incubated overnight at 55°C. DNA, precipitated by adding twice the volume of ethanol, was washed with 70% ethanol before being resuspended in TE buffer (10 mmol/L Tris, 1 nmol/L ethylenediaminetetraacetic acid) for storage at 4°C.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

We used the PCR-RFLP procedure to detect $G_s\alpha$ mutations at the Arg[201] codon (CGT) with strategy primers as reported.[17] Table 1 summarizes the primers used in this study. DNA sequences containing codon 201 of the $G_s\alpha$ gene were amplified using the primers P1 and P2 for 30 cycles (95°C for 60 seconds, 55°C for 60 seconds, and 75°C for 60 seconds). Then 1 μl of the amplified prod-

Table 2. $G_s\alpha$ Mutations at the Arg[201] Codon in Fibrous Dysplasia and Osteofibrous Dysplasia

Case	Age/sex	Diagnosis	Site	$G_s\alpha$ mutation
Control	39/M	Normal bone*	Femur	—
FD72	35/F	Monostotic fibrous dysplasia	Femur	R201H
FD78	15/M	Polyostotic fibrous dysplasia	Femur	R201C
FD99	65/F	Monostotic fibrous dysplasia	Ilium	R201H
FD103	39/F	Monostotic fibrous dysplasia	Femur	R201C
FD104	17/F	Monostotic fibrous dysplasia	Femur	R201H
FD106	17/F	Monostotic fibrous dysplasia	Femur	R201C
FD115	10/F	Monostotic fibrous dysplasia	Femur	R201C
OFD50	3/M	Osteofibrous dysplasia	Fibula	—
OFD56	10/F	Osteofibrous dysplasia	Tibia	—
OFD64	7/M	Osteofibrous dysplasia	Tibia	—
OFD108	13/M	Osteofibrous dysplasia	Tibia	—
OFD137	11/M	Osteofibrous dysplasia	Tibia	—
OFD138	27/M	Osteofibrous dysplasia	Tibia	—
OFD195	16/F	Osteofibrous dysplasia	Tibia	—

R201H, arginine to histidine; R201C, arginine to cysteine.
*Normal bone was obtained from a donor with neither fibrous dysplasia nor osteofibrous dysplasia.
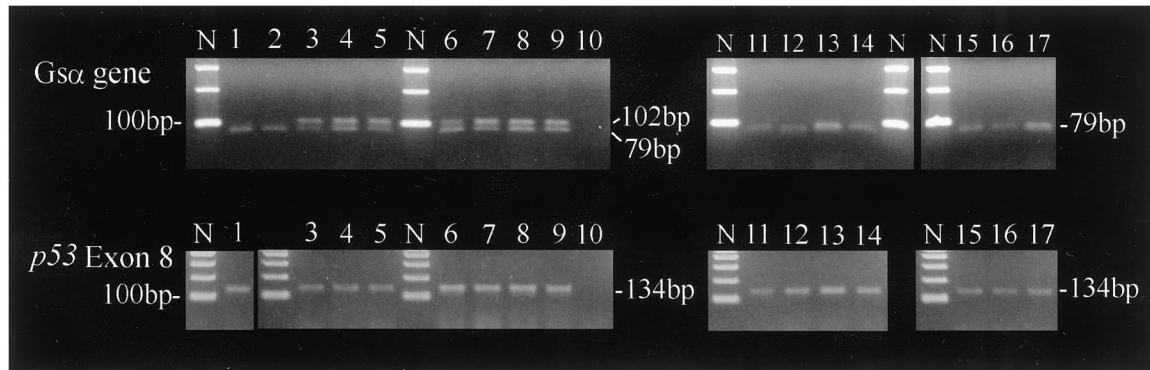
Figure 1. Analysis of G$_S\alpha$ mutations at the Arg[201] codon using RFLP. *Eag*I digested the amplified DNA fragments (102 bp) including codon 201 of the G$_S\alpha$ gene into 79-bp and 23-bp fragments in normal bones (**lanes 1** and **2**) and osteofibrous dysplasia (**lanes 11–17**). The 23-bp band was not visible in this gel, whereas mutant cases of fibrous dysplasia remained undigested (**lanes 3–9**). The *p53* gene exon 8 segments were amplified in each case. The 134-bp segments are shown for each case. PCR using only primers was used as a negative control (**lane 10**). The 100-bp ladder was used as a size marker.

ucts, which had been diluted 50 times, was amplified by means of nested PCR using the mutant primer P3 and the primer P2 for 20 cycles (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds), since the primer P3 creates a new restriction site for *Eag*I (CGGCCG) through the change to G in the first position of codon 200 in the normal allele, thus enabling the detection of a mutation at the first and the second positions of the Arg[201] codon. Substitutions for arginine will not occur even if a point mutation occurs in the third position of codon 201, so this method can be used to detect all of the substitutions for arginine by other amino acids due to a point mutation. *Eag*I digests the 102-bp amplified fragment into two fragments of 79 and 23 bp, thereby revealing the presence of the normal allele, while the mutant allele remains within the undigested 102-bp fragment. Fragments (134 bp) of *p53* gene exon 8 were amplified for 40 cycles (95°C for 1
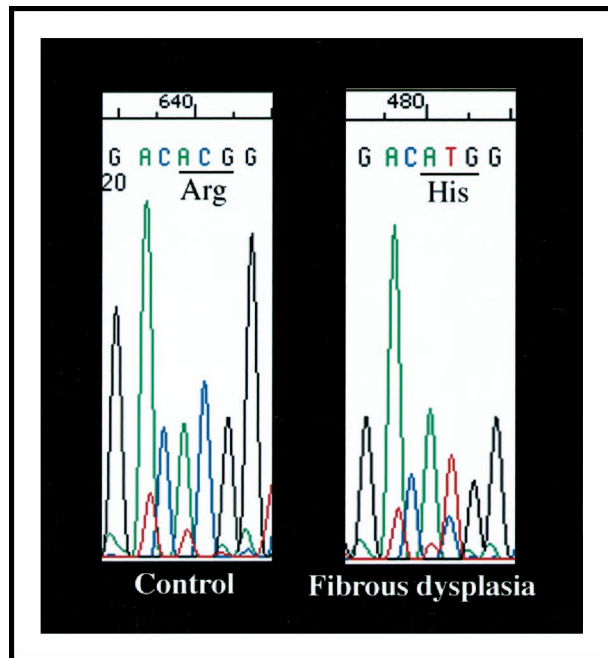

Figure 2. Direct sequencing by the antisense primer P4 was performed on the fibrous dysplasia sample in **lane 5** (Case FD99; 65-year-old female, ilium). The figure shows the G$_S\alpha$ gene antisense sequence and indicates that the second position of codon 201 of the antisense strand was mutated from C to T, this change being the code for histidine instead of arginine (**right**). In normal bone, sequence analysis was performed after reamplified PCR without subsequent endonuclease digestion. The result was that arginine was encoded, and no mutation was seen (**left**).
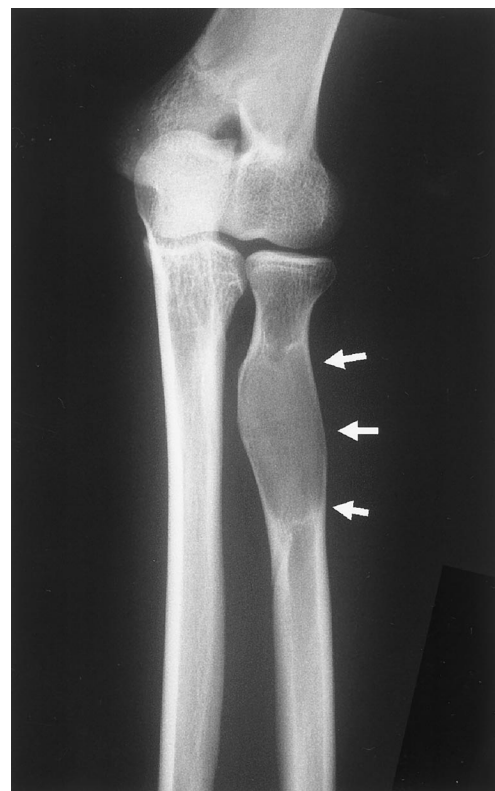

Figure 3. Fibrous dysplasia (12-year-old female, radius). Anteroposterior radiograph reveals a well-circumscribed intramedullary lesion within the proximal radius with a so-called "ground glass" appearance (**white arrows**).
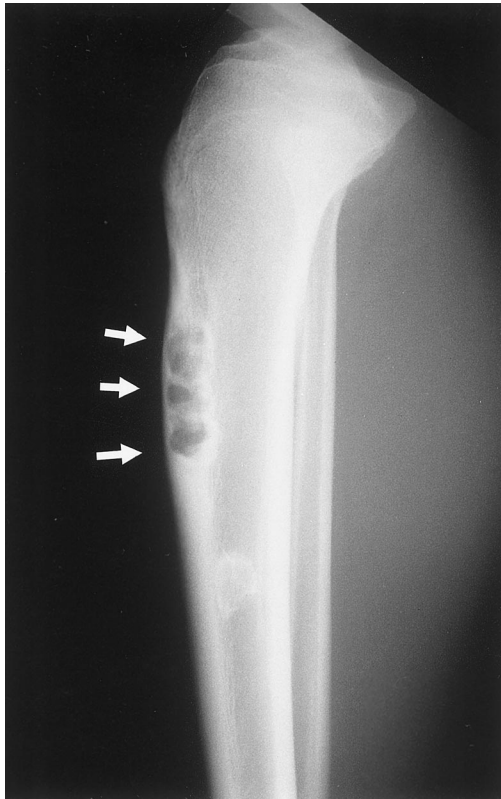
Figure 4. Osteofibrous dysplasia (14-year-old female, tibia). Lateral radiograph reveals an osteolytic intracortical lesion in the tibial shaft with marginal sclerosis (**white arrows**). In this case, an intramedullary lesion that appears to be a bone infarct can be also observed.
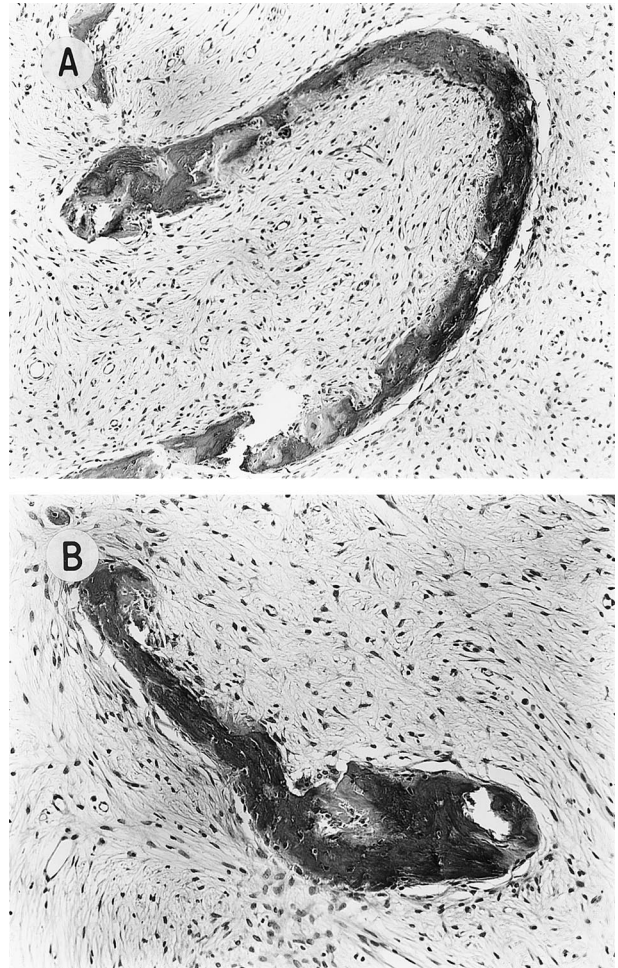

Figure 5. Fibrous dysplasia (15-year-old male, femur). Slender curved trabeculae of bone and cellular proliferation of fibroblast-like cells can be observed (**A**). Note the bone trabeculae have no osteoblastic rimming (**B**). H&E; original magnifications, ×100 (**A**) and ×140 (**B**).

minute, 66°C for 1 minute, and 72°C for 2 minutes) as a positive control to test for the suitability of the respective material for PCR amplification. The DNA bands were analyzed by 3% agarose gel electrophoresis, stained with ethidium bromide, and then photographed.

Direct Sequencing

After samples of the digested products were obtained from agarose gels and reamplified by primers P3 and P4 for 20 cycles (95°C for 30 seconds and 55°C for 30 seconds), the amplified product was purified by centrifugal filter devices of Microcon (Millipore, Bedford, MA). After purification, direct sequencing was carried out by the dideoxy chain termination method using a Perkin Elmer ABI Prism 310 sequence analyzer (Perkin Elmer, Foster City, CA). The primer used for direct sequencing was the antisense primer P4. Because codon 201 is located next to the 3′ end of the sense primer P3, it was not technically feasible to carry out direct sequencing with the sense primer P3 using the dideoxy chain termination method.

Results

Table 2 summarizes the data. The $G_S\alpha$ point mutations at the Arg[201] codon occurred in all seven cases of fibrous dysplasia, comprising four cases of G-to-A transition in

the first position and three cases of C-to-T transition in the second position at codon 201, corresponding to the previously reported Arg-to-Cys and Arg-to-His substitutions respectively. On the other hand, all seven cases of osteofibrous dysplasia as well as the normal bone used as a control showed no mutation in the $G_S\alpha$ gene at the Arg[201] codon (Figures 1 and 2).

Discussion

Clinically, fibrous dysplasia differs from osteofibrous dysplasia with regard to the affected site, affected age group, radiographic appearance and clinical course (Figures 3 and 4).[15,18,19] These findings indicate that fibrous dysplasia differs in nature from osteofibrous dysplasia. However, because their histological features are often similar,[18] the question of whether osteofibrous dysplasia is actually a separate entity or simply a variant of fibrous dysplasia (Figures 5 and 6) remains controversial.[16]

In the present study, we used PCR-RFLP and direct sequencing analysis to detect the occurrence of $G_S\alpha$ mutations at the Arg[201] codon in fibrous dysplasias and
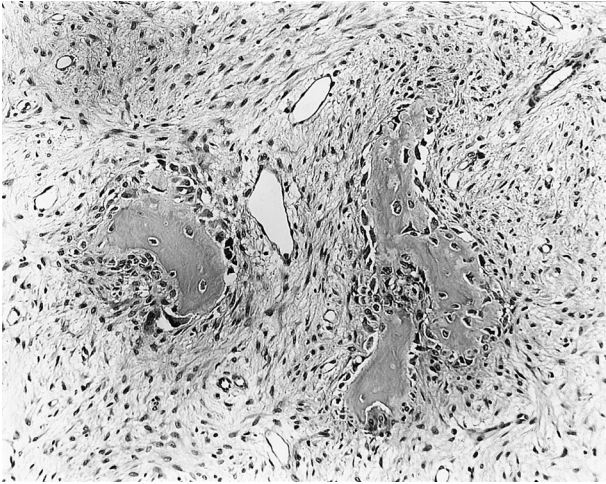
Figure 6. Osteofibrous dysplasia (13-year-old female, tibia). Microscopic features are characterized by woven bone trabeculae with rimming of osteoblasts and a cellular proliferation of fibroblast-like cells. H&E; original magnification, ×140.

osteofibrous dysplasias using formalin-fixed, paraffin-embedded decalcified tissue. A variety of techniques has been developed for detecting $G_S\alpha$ mutations. This method of PCR-RFLP analysis can detect $G_S\alpha$ mutations at the Arg^{201} codon without using any radioactive material, even when the mutations are present in low abundance.[17]

In addition to the substitution of either Cys or His at $Arg,^{20,15,11}$ the substitution of Ser has also been detected.[17] These somatic mutations in the $G_S\alpha$ gene lead to constitutively activated adenylate cyclase activity,[20] elevated cAMP levels, and increased proliferation of hormonally responsive osteoblastic cells, resulting in the overproduction of a disorganized collagenous matrix.[5,8–11] Thus, both polyostotic and monostotic fibrous dysplasia seem to result from the abnormal proliferation of mesenchymal osteoblast progenitor cells with $G_S\alpha$ mutation. On the other hand, the $G_S\alpha$ mutation may stimulate osteoblastic cell proliferation of early immediate genes, including c-*fos*. The products of c-*fos* proto-oncogene have been associated with the control of bone cell proliferation and differentiation.[21] The increased production of cAMP in bone cells with $G_S\alpha$ mutations most likely leads to elevated c-*fos* expression,[22] which also plays an important part in the development of fibrous dysplasia.[23] There is another possible mutation site in the $G_S\alpha$ gene at Gln^{227} that has been shown to result in activation. A substitution of either Leu or Arg at Gln^{227} has been found in certain endocrine tumors,[24,25] but has not yet been identified in association with bone. On the other hand, mutation in the $G_S\alpha$ gene at Gln^{227} cannot be ruled out by the analysis that was performed in this study.

Alman et al[26] demonstrated $G_S\alpha$ mutation in all four of their cases of fibrous dysplasia but not in their one case of osteofibrous dysplasia. We were also able to detect $G_S\alpha$ point mutations at the Arg^{201} codon, comprising substitutions of Arg-to-Cys or Arg-to-His as others have previously reported,[5,11] in all our seven cases of fibrous dysplasia, but not in any of our cases of osteofibrous

dysplasia. We cannot deny the possibility that there were simply not enough mutant cells present to be detected by this method and that there may be a potential problem with recovery of an adequate quality of intact DNA due to decalcification in hydrochloric acid. However, our data support their conclusion that fibrous dysplasia and osteofibrous dysplasia are distinct pathological entities with a different molecular pathobiology.

Komiya et al[27] surmised that abnormalities in the blood circulation within the periosteum are probably behind the pathogenesis of osteofibrous dysplasia. Pathological periosteum may stimulate the production of excessive osteoclasts. The tumorous condition of osteofibrous dysplasia can probably be attributed to dysregulation of bone remodeling, in which osteoclastosis is dominant to osteogenesis.

According to the report of Sweet et al,[28] when comparing fibrous dysplasia and osteofibrous dysplasia, immunohistochemical staining of cytokeratin (AE1/AE3 + CK-1) seemed to be helpful for distinguishing between these lesions, because isolated cytokeratin-positive cells were seen in the stroma of 28 of 30 cases of osteofibrous dysplasia (93%), but not in any of the 47 cases of fibrous dysplasia (43 monostotic and 4 polyostotic cases).

Not only the clinical, pathological, and immunohistochemical differences, but also the presence or absence of $G_S\alpha$ mutations at the Arg^{201} codon would seem to suggest that fibrous dysplasia is a different lesion from osteofibrous dysplasia.

We demonstrated $G_S\alpha$ mutation in a series of fibrous dysplasia and osteofibrous dysplasia using paraffin-embedded decalcified tissue by means of PCR-RFLP analysis. $G_S\alpha$ mutation at the Arg^{201} codon was seen in all of the fibrous dysplasia cases, but not in any of the osteofibrous dysplasia cases. Our data suggest that fibrous dysplasia and osteofibrous dysplasia are of a different pathogenesis. The detection of $G_S\alpha$ mutation at the Arg^{201} codon would therefore seem to be quite useful for distinguishing between fibrous dysplasia and osteofibrous dysplasia when making a pathological diagnosis.

Acknowledgments

References

1. Lichtenstein L: Polyostotic fibrous dysplasia. Arch Surg 1938, 36: 874–898
2. Lichtenstein L, Jaffe HL: Fibrous dysplasia of a bone: condition affecting one, several or many bones, graver cases of which may present abnormal pigmentation of skin, premature sexual development, hyperthyroidism or still other extraskeletal abnormalities. Arch Pathol 1942, 33:777
3. Arbright F, Bulter AM, Hampton AO, Smith P: Syndrome characterized by osteitis fibrosa disseminata, areas of pigmentation and endocrine dysfunction with precocious puberty in females. N Engl J Med 1937, 216:727–746

4. Danon M, Crawford JD: The McCune-Albright syndrome. Ergeb inn Med Kinderheild 1987, 55:81–115

5. Riminucci M, Liu B, Corsi A, Shenker A, Spiegel AM, Robey PG, Bianco P: The histopathology of fibrous dysplasia of bone in patients with activating mutations of the Gs alpha gene: site-specific patterns and recurrent histological hallmarks. J Pathol 1999, 187:249–258

6. Weinstein LS, Shenker A, Gejman PV, Merino MJ, Friedman E, Spiegel AM: Activating mutations of the stimulatory G protein in the McCune-Albright syndrome. N Engl J Med 1991, 325:1688–1695

7. Shenker A, Weinstein LS, Sweet DE, Spiegel AM: An activating Gs alpha mutation is present in fibrous dysplasia of bone in the McCune-Albright syndrome. J Clin Endocrinol Metab 1994, 79:750–755

8. Schwindinger WF, Francomano CA, Levine MA: Identification of a mutation in the gene encoding the alpha subunit of the stimulatory G protein of adenylyl cyclase in McCune-Albright syndrome. Proc Natl Acad Sci USA 1992, 89:5152–5156

9. Malchoff CD, Reardon G, MacGillivray DC, Yamase H, Rogol AD, Malchoff DM: An unusual presentation of McCune-Albright syndrome confirmed by an activating mutation of the Gs alpha-subunit from a bone lesion. J Clin Endocrinol Metab 1994, 78:803–806

10. Marie PJ, de Pollak C, Chanson P, Lomri A: Increased proliferation of osteoblastic cells expressing the activating Gs alpha mutation in monostotic and polyostotic fibrous dysplasia. Am J Pathol 1997, 150:1059–1069

11. Riminucci M, Fisher LW, Shenker A, Spiegel AM, Bianco P, Gehron Robey P: Fibrous dysplasia of bone in the McCune-Albright syndrome: abnormalities in bone formation. Am J Pathol 1997 151:1587–1600

12. Campanacci M: Osteofibrous dysplasia of long bones, a new clinical entity. Ital J Orthop Traumatol 1976, 2:221–237

13. Alguacil-Garcia A, Alonso A, Pettigrew NM: Osteofibrous dysplasia (ossifying fibroma) of the tibia and fibula and adamantinoma: a case report. Am J Clin Pathol 1984, 82:470–474

14. Keeney GL, Unni KK, Beabout JW, Pritchard DJ: Adamantinoma of long bones: a clinicopathologic study of 85 cases. Cancer 1989, 64:730–737

15. Markel SF: Ossifying fibroma of long bone: its distinction from fibrous dysplasia and its association with adamantinoma of long bone. Am J Clin Pathol 1978, 69:91–97

16. Park YK, Unni KK, McLeod RA, Pritchard DJ: Osteofibrous dysplasia: clinicopathologic study of 80 cases. Hum Pathol 1993, 24:1339–1347

17. Candeliere GA, Roughley PJ, Glorieux FH: Polymerase chain reaction-based technique for the selective enrichment and analysis of mosaic arg201 mutations in G alpha s from patients with fibrous dysplasia of bone. Bone 1997, 21:201–206

18. Sakamoto A, Oda Y, Iwamoto Y, Tsuneyoshi M: A comparative study of fibrous dysplasia and osteofibrous dysplasia with regard to expressions of c-fos and c-jun products and bone matrix proteins: a clinicopathological review and immunohistochemical study of c-fos, c-jun, type i collagen, osteonectin, osteopontin and osteocalcin. Human Pathol 1999, 30:1418–1426

19. Wang JW, Shih CH, Chen WJ: Osteofibrous dysplasia (ossifying fibroma of long bones): a report of four cases and review of the literature. Clin Orthop 1992, 278:235–243

20. Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L: GTPase inhibiting mutations activate the alpha chain of Gs, and stimulate adenylyl cyclase in human pituitary tumours. Nature 1989, 340:692–696

21. Machwate M, Jullienne A; Temporal variation of c-fos proto-oncogene expression during osteoblast differentiation, and osteogenesis in developing rat bone. J Cell Biochem 1995, 57:62–70

22. Gaiddon C, Boutillier AL, Monnier D, Mercken L, Loeffler JP: Genomic effects of the putative oncogene G alpha s: chronic transcriptional activation of the c-fos proto-oncogene in endocrine cells. J Biol Chem 1994, 269:22663–22671

23. Candeliere GA, Glorieux FH, Prud'homme J, St-Arnaud R: Increased expression of the c-fos proto-oncogene in bone from patients with fibrous dysplasia. N Engl J Med 1995, 332:1546–1551

24. Vallar L: GTPase-inhibiting mutations activate the alpha-chain of Gs in human tumours. Biochem Soc Symp 1990, 56:165–170

25. Clementi E, Malgaretti N, Meldolesi J, Taramelli R: A new constitutively activating mutation of the Gs protein alpha subunit-gsp oncogene is found in human pituitary tumours. Oncogene 1990, 5:1059–1061

26. Alman BA, Greel DA, Wolfe HJ: Activating mutations of Gs protein in monostotic fibrous lesions of bone. J Orthop Res 1996, 14:311–315

27. Komiya S, Inoue A: Aggressive bone tumorous lesion in infancy: osteofibrous dysplasia of the tibia and fibula. J Pediatr Orthop 1993, 13:577–581

28. Sweet DE, Vinh TN, Devaney K: Cortical osteofibrous dysplasia of long bone and its relationship to adamantinoma: a clinicopathologic study of 30 cases. Am J Surg Pathol 1992, 16:282–290